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Letter to the Editor

AllergoOncology: Expression platform development and functional profiling of an anti-HER2 IgE antibody

Summary

Efficient strategies for generation of recombinant IgE class antibodies at high-enough yields for pre-clinical screening and mechanistic evaluations remain challenging. We present a transient expression application for the rapid production of recombinant IgE, exemplified with the cloning and functional characterization of a humanised IgE antibody bearing the variable region sequences of the anti-HER2/*neu* antibody trastuzumab. Material was generated with high transfection efficiency, in small culture volumes within 7-9-days from design to purified material, and at sufficient yields for functional studies. The antibody conserved the trastuzumab Fab-mediated recognition of the target antigen, and IgE Fc attributes to recognise FcεRs and to potentiate immune cell-mediated effector functions. Moreover, mast cell and basophil activation tests confirmed lack of activation with IgE in the absence of cross-linking stimuli, supporting potential safe administration in humans. Our study facilitates fast generation of IgE antibodies for speedy screening and functional characterisation and can be readily applied to the design and evaluation of IgE antibodies in Allergy and AllergoOncology.

To the Editor,

Monoclonal antibodies approved for the treatment of cancer belong to the IgG class (most often IgG1). However, IgG has limited tissue half-life (2-3 days), relatively low affinity for cognate Fc receptors and the disadvantage of interaction with inhibitory Fcγ receptors, abundant in the tumour microenvironment. Conversely, IgE class antibodies may offer new options for cancer therapy, based on high affinity for cognate Fcε receptors expressed on different, often tumour-resident, immune effector cells such as macrophages and mast cells, and lack of inhibitory Fc receptors¹. IgE-mediated tissue surveillance functions known to potentiate “allergic” or “pathogen/parasite-clearing” immunity could be re-directed against tissue-resident tumours^{2,3}. IgE antibodies recognising the tumour-associated antigen Folate Receptor α (FRα) induced superior immune responses in disparate *in vivo* models, highlighting potential opportunities for FRα-expressing ovarian carcinomas². In breast cancer, *in vitro* studies of trastuzumab (IgG1) and an engineered trastuzumab IgE recognising the tumour-associated antigen HER2/*neu* indicated that IgE could complement or possibly improve the clinical

performance of trastuzumab⁴. The first-in-class IgE antibody (MOv18) is undergoing an early phase clinical trial in patients with FR α -expressing carcinomas (NCT02546921, www.clinicaltrials.gov).

Despite considerable progress, production of monoclonal antibodies remains time-consuming and labour-intensive. One reason is the requirement for expression of heavy (HC) and light chains (LC) in a controlled manner, usually cloned in separate expression vectors using enzymatic restriction digestion and ligation. This introduces experimental variability in expression procedures and is often inefficient. These limitations also concern the study of anti-allergen IgE, where Fabs rather than full-length antibodies are commonly expressed and evaluated⁵⁻⁷. Therefore, antibody cloning systems are moving towards utilisation of single dual-expression plasmids (*e.g.* pcDNA3.3 and pVito1 hygro-mcs), to increase antibody production⁸.

Building upon ours and others' previous methodologies, we report the efficient transient expression and functional evaluation of IgE, exemplified using the variable region sequences of trastuzumab and human IgE constant regions (anti-HER2 IgE).

We employed polymerase incomplete primer extension (PIPE) PCR cloning and enzyme-free assembly of DNA fragments. The amino acid sequences of trastuzumab variable light (VL) and heavy (VH) chain regions were manually codon-optimized for a human expression host and cloned into a pVito1-hygro-mcs dual expression vector containing pre-cloned cassettes of the human epsilon HC and kappa LC using PIPE PCR cloning methodology (Figure 1A)⁸. PIPE PCR was performed using the pVito1 plasmid to generate linear PCR fragments with 5' PIPE overhangs, and trastuzumab variable region fragments to derive VL and VH region fragments with 5' PIPE overhangs (DNA fragment sizes by agarose gel electrophoresis, Figure 1B).

Expression was conducted transiently in human embryonic kidney (Expi293F) cells without antibiotic selection, in 30mL serum-free suspension cultures (Figure 1C). Variable region codon optimisation enhanced antibody yields (~7-fold) (Figure 1D). Peak antibody concentrations (70-80 μ g/mL) were achieved within 7-9 days (supernatants harvested after 7 days, Figure 1C, 1E). After purification, total yields were 60 μ g/mL (>85% purification efficiencies) (Figure 1F). SDS-PAGE of purified antibodies under non-reducing conditions showed a 250kDa band, likely reflecting high antibody glycosylation), and reducing conditions revealed two signals (75kDa (HC), 25kDa (LC)), and a slight signal (100kDa) likely representing different HC glycoforms (Figure 1G). HPLC analysis demonstrated assembly of monomeric IgE (Figure 1H).

Like trastuzumab, anti-HER2 IgE recognized HER2/*neu*-overexpressing (BT-474, ZR75-30) breast cancer cells and moderately-expressing MCF-10 normal breast cells, and its HER2 antigen recognition kinetic profile on tumour cells was comparable to trastuzumab (Figure 2A). Anti-HER2 IgE and

trastuzumab similarly-restricted breast cancer cell viability, and epidermal growth factor signalling, while addition of antibodies together did not improve HER2 signalling inhibition (Figure 2B-C). Consistent with FcεR-binding MOv18 IgE^{1,2} (produced in SP2/0 cells), anti-HER2 IgE recognised RBL SX-38 rat basophilic leukaemia cells, expressing the human tetrameric FcεRI(αβγ2), and human U937 monocytes expressing the low-affinity IgE receptor FcεRII/CD23 upon IL-4 stimulation (Figure 2D). Similar to MOv18 IgE, anti-HER2 IgE recognised FcεRI-expressing human primary monocytes and anti-HER2 IgE binding kinetics to RBL SX-38 were comparable to those of MOv18 IgE (Figure 2D).

Anti-HER2 IgE induced >2-fold higher ADCC of HER2-overexpressing breast cancer cells by unstimulated and IL-4 stimulated U937 effector cells compared with isotype controls (Figure 2E). Anti-HER2 IgE triggered higher ADCC against breast cancer cells by peripheral blood mononuclear cells (PBMCs from human volunteers, HV, Figure 2E), and >2-fold higher ADCC by RBL SX-38 cells (Figure 2F) compared with isotype controls (see Supplementary file).

Anti-HER2 IgE induced degranulation of RBL SX-38 cells when cross-linked by polyclonal anti-IgE on the cell surface (left), or by HER2-expressing tumour cells (right), but not without cross-linking stimulus or with recombinant monomeric antigen (HER2 ectodomain (ECD)) (Figure 2G). In basophil activation tests (BAT) conducted in unfractionated human blood, anti-HER2 IgE did not induce basophil activation, monitored by upregulation of the activation marker CD63 (Figure 2H-I). Mast cell and basophil tests therefore confirm lack of activation with IgE in the absence of cross-linking stimuli⁹, supporting potential safe administration in human circulation.

IgE immunotherapy may offer a promising approach for cancer treatment, contributing to the emerging field of AllergoOncology, focused on dissecting interplay between IgE, allergy and malignancy. Development of efficient platforms for speedy generation of full-length IgE at appreciable yields for numerous evaluations to expedite the field, remains challenging. Our herein-described multi-gene cloning, enzyme-free assembly system for rapid expression of functionally-active antibody, within 7-9 days from transfection to purification in serum-free cultures (2mg purified material from 30mL), readily-established even in “small” environments, surpassing previous platforms in expression efficiency, speed (7-9 days vs 4-6 weeks) and yields (70-80mg/mL vs <20-25mg/mL)⁴, meets these challenges. IgE maintained Fab- and Fc-mediated properties, including antigen and receptor binding, ADCC and degranulation, contributing to the most important/prominent antibody functionalities. These suggest that under conditions akin to those of tumours, when encountering high levels of HER2-expressing cancer cells, anti-HER2 IgE may trigger mast cell activation and anti-tumour effector functions. Importantly, the lack of anti-HER2 IgE blood basophil activation points to diminishing potential safety concerns associated with using IgE class antibodies in cancer immunotherapy. Our report of transient cloning and rapid antibody production greatly facilitates the study of IgE structural

and immune functional attributes and may find numerous applications in allergy, biotechnology and immunology-related fields.

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CONFLICTS OF INTEREST

S.N. Karagiannis and J.F. Spicer are founders and shareholders of IGEM Therapeutics Ltd. S.N. Karagiannis holds a patent on anti-tumour IgE antibodies. All other authors have declared that no conflict of interest exists.

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AUTHOR CONTRIBUTIONS

KMI, SNK, and AJNT conceived and designed the study. KMI, SC, SM, JF-S, HJB helped with the development of the methodology. KMI, JF-S, SC, SM, HJB, CS, HSS, LMM and DHJ generated reagents, acquired the data or helped with the data analysis and interpretation. KMI, JF-S, SC, SM, HJB, LMM, HS, DHJ, JFS, EJ-J, AJNT, and SNK discussed and interpreted the data and edited the manuscript. SNK supervised the study, led and coordinated the project. KMI and SNK wrote the manuscript.

REFERENCES

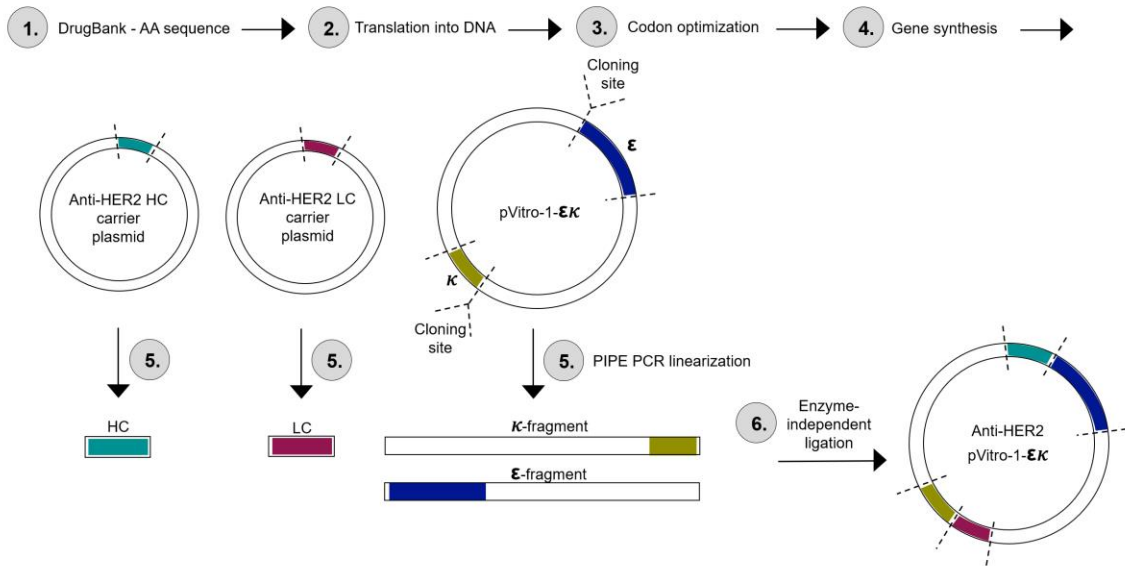
1. Josephs DH, Bax HJ, Dodev T, et al. Anti-Folate Receptor-alpha IgE but not IgG Recruits Macrophages to Attack Tumors via TNFalpha/MCP-1 Signaling. *Cancer Res.* 2017;77:1127-1141.
2. Josephs DH, Nakamura M, Bax HJ, et al. An immunologically relevant rodent model demonstrates safety of therapy using a tumour-specific IgE. *Allergy.* 2018;73:2328-2341.
3. Platzer B, Elpek KG, Cremasco V, Baker K, Stout MM, Schultz C, Dehlink E, Shade KT, Anthony RM, Blumberg RS, Turley SJ, Fiebiger E. IgE/FcεRI-Mediated Antigen Cross-Presentation by Dendritic Cells Enhances Anti-Tumor Immune Responses. *Cell Rep.* 2015 doi: 10.1016/j.celrep.2015.02.015.
4. Karagiannis P, Singer J, Hunt J, et al. Characterisation of an engineered trastuzumab IgE antibody and effector cell mechanisms targeting HER2/neu-positive tumour cells. *Cancer Immunol Immunother.* 2009;58:915-930.
5. Mitropoulou AN, Bowen H, Dodev TS, et al. Structure of a patient-derived antibody in complex with allergen reveals simultaneous conventional and superantigen-like recognition. *Proc Natl Acad Sci U S A.* 2018;115:E8707-E8716.
6. Niemi M, Janis J, Jylha S, et al. Characterization and crystallization of a recombinant IgE Fab fragment in complex with the bovine beta-lactoglobulin allergen. *Acta Crystallogr Sect F Struct Biol Cryst Commun.* 2008;64(Pt 1):25-28.
7. Niemi M, Jylha S, Laukkanen ML, et al. Molecular interactions between a recombinant IgE antibody and the beta-lactoglobulin allergen. *Structure.* 2007;15:1413-1421.
8. Ilieva KM, Fazekas-Singer J, Achkova DY, et al. Functionally Active Fc Mutant Antibodies Recognizing Cancer Antigens Generated Rapidly at High Yields. *Front Immunol.* 2017;8:1112.
9. Rudman SM, Josephs DH, Cambrook H, et al. Harnessing engineered antibodies of the IgE class to combat malignancy: initial assessment of FcγεRI-mediated basophil activation by a tumour-specific IgE antibody to evaluate the risk of type I hypersensitivity. *Clin Exp Allergy.* 2011;41:1400-1413.

FIGURE LEGENDS

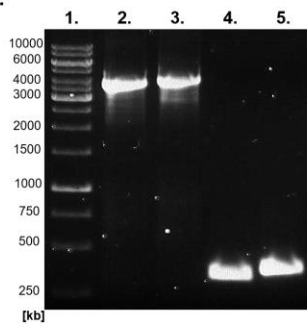
FIGURE 1. Anti-HER2 IgE cloning and generation. A. Cloning strategy. 1-4: Variable region DNA sequence generation. 5: Trastuzumab variable region plasmids, pVitro1 plasmid with kappa/epsilon constant chains linearized (PIPE PCR), generating 4 fragments with 5' PIPE overhangs. 6: Linear fragments assembled non-enzymatically (pVitro-1- $\epsilon\kappa$). B. Agarose gel electrophoresis (PIPE fragments). 1: DNA ladder, 2: ϵ -fragment (4099bp), 3: κ -fragment (4119bp), 4: LC (364bp), 5: HC (408bp). C. Expression strategy. D. 7-day yields following codon-optimization (representative). Expression before (E), after (F) purification (\pm SD, representative of n=2). G. SDS-PAGE: 1: protein standard, 2: non-reducing, 3: reducing conditions. H. HPLC trace after size exclusion chromatography.

FIGURE 2. Anti-HER2 IgE functional characterization. (A) Flow cytometric binding/kinetic profiles to breast cancer and normal breast (MCF-10) cells. IgE reduced breast cancer cell viability (B), and HER2/*neu* signalling (n=2) (C). D: Flow cytometric binding/kinetic profiles of IgE to human Fc ϵ R-expressing: RBL SX-38 mast cells, U937 monocytes, human monocytes (healthy volunteers, HV) (anti-FR α IgE (MOV18): control). E-F. IgE-mediated % tumour cell killing (\pm SD): (E) by U937 (n=3), human (HV) PBMC (n=6); (F) by RBL SX-38. G. RBL SX-38 degranulation experiments (β -hexosaminidase release, Triton X-100-lysis (Tx100): 100% granule release, representative of n=2). G-H. Anti-HER-2 IgE stimulation in basophil activation test (BAT).

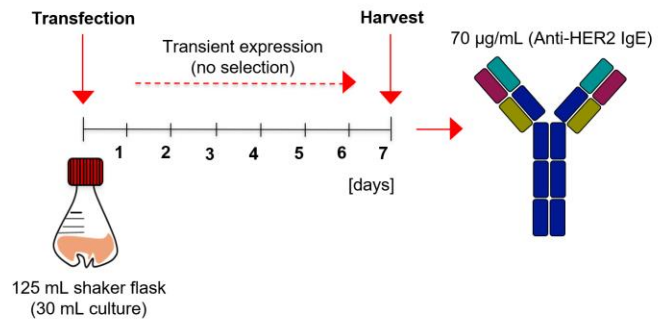
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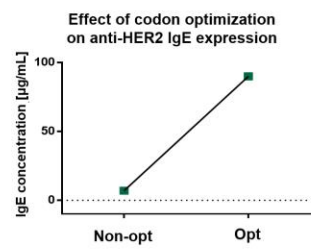
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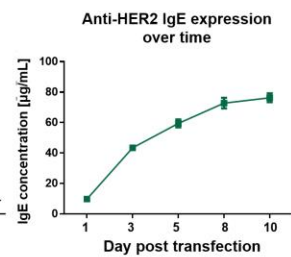
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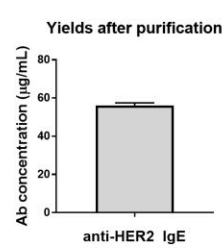
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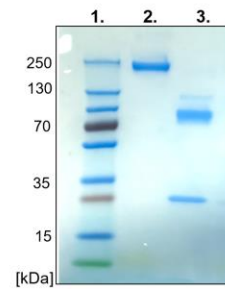
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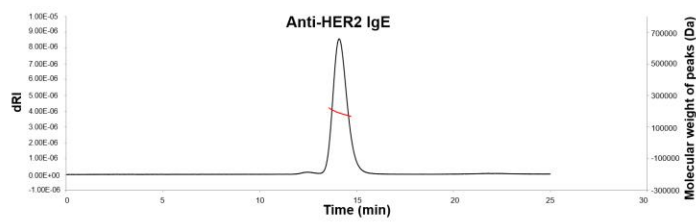
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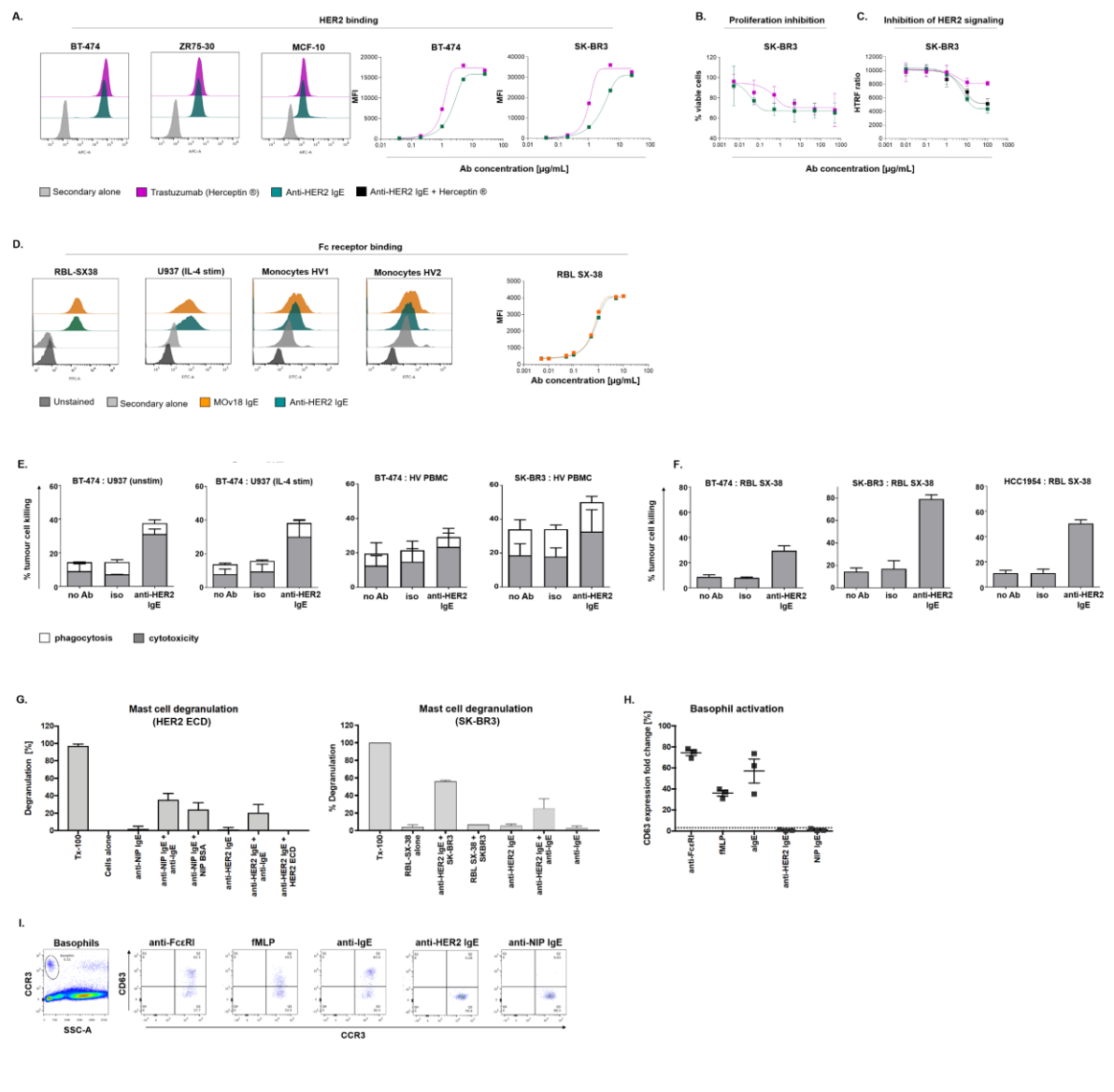


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Supplementary Materials and Methods

AllergoOncology: Expression platform development and functional profiling of an anti-HER2 IgE antibody

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Generation and expression of anti-HER2 IgE

Antibody cloning: We employed polymerase incomplete primer extension (PIPE) PCR cloning and enzyme-free assembly of DNA fragments. The amino acid sequences of the trastuzumab heavy and light variable regions were obtained from the DrugBank database (www.drugbank.ca), translated in nucleotide sequences and manually codon optimized for a human expression host. Optimized sequences were synthesized using GeneArt Gene Synthesis (Thermo Fischer Scientific UK). The DNA sequences of the variable regions were previously reported¹. The variable region fragments of trastuzumab were cloned into pVito1-hygro-mcs dual expression vector containing pre-cloned cassettes of the human epsilon chain constant region and kappa light chain constant region cassettes using the polymerase incomplete primer extension (PIPE) PCR cloning as previously described². The PIPE PCR primers used for the fragment amplification are listed in Supplementary Table S1. Briefly, PIPE PCR was performed using pVito1 plasmid as a template in order to generate two linear PCR fragments with 5' PIPE overhangs, and commercially-generated trastuzumab variable region fragments, in order to generate a variable light (VL) and heavy (VH) region fragments with 5' PIPE overhangs. Correct sizes of the DNA fragments were confirmed with agarose electrophoresis. Anti-HER2 IgE expression was carried out transiently in Expi293F cells using small volume suspension culture (30 mL) for ca. 7-9 days to achieve peak antibody concentrations of ca. 70-80 µg/mL in the culture supernatant.

Purification and HPLC: The antibody was purified using KappaSelect affinity chromatography (GE Healthcare), concentrated with Amicon centrifugal filters, (MWCO 10,000 kDa, Merck Millipore, Germany) and analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using pre-cast 4-16% gels (Bio-Rad). Before performing analytical high-performance liquid chromatography (HPLC), HER2-IgE was subjected to size-exclusion chromatography (SEC) on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare, USA) equilibrated with PBS plus 200 mM NaCl, and fractions pooled and concentrated. HPLC SEC combined with multi-angle light scattering was

performed to confirm the molar mass of the monomeric IgE collected resulting from preparative SEC. HPLC (Shimadzu prominence LC20, Japan) was equipped with MALS (WYATT Heleos Dawn8+ plus QELS; software ASTRA 6), refractive index detector (RID-10A, Shimadzu) and a diode array detector (SPD-M20A, Shimadzu). The column (Superdex 200 10/300 GL, GE Healthcare, USA) was equilibrated with PBS plus 200 mM NaCl (pH 7.4) as running buffer. Prior to analysis, the IgE sample was centrifuged (17,000 g, 10 min, 20°C) and filtered (0.1 mm Ultrafree-MC filter, Merck Millipore, Germany). Experiments were performed at a flow rate of 0.75 mL min⁻¹ at 25°C. The proper performance of molar mass calculation by MALS was verified by the determination of a sample of bovine serum albumin.

Isolation of Human Immune Cells

Human samples were collected with informed written consent, in accordance with the Helsinki Declaration. Study design was approved by the Guy's Research Ethics Committee (REC No 07/H0804/131), Guy's and St. Thomas' NHS Foundation Trust. Peripheral blood was also obtained through the UK National Health System (NHS) Blood and Transplant system from anonymous donor leukocyte cones. Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll® Paque PLUS (GE Healthcare) density gradient centrifugation. Red blood cells were lysed from extracted PBMCs using RBC Lysis buffer (Biolegend).

Assessment of antibody binding to cells by flow cytometric evaluations

Recognition of the target antigen on tumour cells by anti-HER2 antibodies and of the human FcεRI on rat basophilic leukaemia RBL SX-38 cells (which stably express the human tetrameric (αβγ2) high-affinity IgE receptor, FcεRI) by anti-HER2 and anti-Folate Receptor alpha (FRα)-specific chimeric MOv18 IgE, were assessed by flow cytometric binding assays. Adherent cell lines were detached using 0.25% trypsin-EDTA (Thermo Fisher Scientific UK). Human healthy volunteer peripheral blood mononuclear cells were extracted using Ficoll-Paque PLUS (GE Healthcare) density centrifugation. Cells were re-suspended in 2% foetal bovine serum in phosphate buffered saline (FACS buffer) and incubated in 96-well round-bottom plates (2x10⁵ tumour cells per well; 1x10⁵ RBL SX-38 cells per well) on ice in the presence of primary antibodies (for serial dilutions, concentration range 0.04 to 25 µg/mL (antibody binding to HER2 over-expressing cancer cell lines) and 0.005 to 10 µg/mL (binding to RBL SX-38) for 30 min. The cells were washed and incubated with secondary Alexa Fluor 647 (AF647)-conjugated anti-human kappa chain antibody (Southern Biotech, UK) or FITC-conjugated goat anti-human IgE (Vector Laboratories, UK). All flow cytometric evaluations were conducted on a BD

FACSCanto™ II or BD LSRFortessa™ cell analysers (BD Biosciences, UK). The data were analysed using FlowJo software version 10.

Cell proliferation assays

To determine the effect of anti-HER2 IgE and IgG1 (trastuzumab) antibody on cell proliferation, 1,000 SK-BR3 breast cancer cells per well were plated in a 96-cell well tissue culture plates in complete medium and treated with serially diluted antibodies (concentration range 0.005-500 µg/mL). Cell proliferation was assessed after 120 hours using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega), according to the manufacturer's instructions. Cell densities were measured as previously described¹ using a Fluostar® Omega Spectrophotometer (BMG Labtech).

Human epidermoid growth factor 2 (HER2, Erbb2) signalling assays

The ability of anti-HER2 IgE to interfere with human epidermoid growth factor 2 (HER2, Erbb2) signalling activity on tumour cells was detected using a Phospho-HER2 (Tyr1221/1222) cellular kit (cisbio, UK) according to the manufacturer's instructions. Briefly, 50,000 SK-BR3 breast cancer cells per well were plated in 96-well plates and allowed to adhere overnight at 37° C, 5% CO₂. The following day cells were incubated for 30 min with anti-HER2 IgE and/or Trastuzumab IgG at different concentrations and subsequently stimulated with 0.05 µg/ml hEGF (human epidermal growth factor) for 10 min at 37 °C, 5% CO₂. Media were then removed, cells were lysed with 50 µL of Lysis buffer for 30 min at RT under gentle shaking, and 16 µL of lysate was transferred into a 96 low-volume white microplate for detection and 4 µL of the HTRF phospho-HER2 detection antibodies were added. After a 4-hour incubation, the levels of Phospho-HER2 signal were recorded by measuring fluorescent emission at 665nm and 620nm in a Fluostar® Omega microplate reader.

Antibody-dependent cellular cytotoxicity/phagocytosis (ADCC/ADCP) assay with primary human PBMC effector cells, U937 effector cells

ADCC/ADCP was quantified using a three-colour flow cytometric assay, modified from previously described methods^{3,4}. Briefly, BT-474 and SK-BR3 breast cancer cells were pre-stained with CFSE cell tracking dye (Life Technologies) 16 hours before the assay. Next, the cancer cells were pre-incubated with 10 µg/mL anti-HER2 IgE or isotype control anti-NIP 228 IgE for 30 min at 37° C, washed and mixed with freshly isolated human PBMCs at an E:T (effector to target) ratio ca. 20:1 or U937 monocytic cells (non-stimulated or pre-stimulated with IL-4) (selected from conditions tested, see Supplementary

Figure S1) at E:T 3:1 in RPMI GlutaMAX™ (Thermo Fischer Scientific) containing 2% FCS (Thermo Fischer Scientific). The cells were co-incubated in 5 mL test tubes (BD Biosciences) for 3 hours at 37°C, 5% CO₂. After the incubation, the primary monocytes/U937 cells were stained with anti-CD89 APC-conjugated antibody (BioLegend) on ice for 30 min. Dead cells were identified using DAPI live/dead dye (Life Technologies). The flow cytometric analyses were performed on a BD FACSCanto™ and data were analysed by evaluating two colour dot plots as previously described^{3,4} (examples in Supplementary Figure S2) using the FlowJo software version 10.

Antibody-dependent cellular cytotoxicity assay with RBL SX-38 effector cells

Rat basophilic leukaemia RBL SX-38, stably expressing the human tetrameric ($\alpha\beta\gamma 2$) high-affinity IgE receptor, Fc ϵ RI, were used as effector cells in a cytotoxicity (ADCC) assay. For experiments targeting HCC1954 and SK-BR3 breast cancer cells, target cells were stained with CFSE cell tracking dye (FITC channel) 16 hours before the assay. Next, the target cells were pre-incubated with 0.5 μ g IgE per test for 30 min at 37°C and subsequently incubated with RBL SX-38 effector cells (E:T 10:1) for 2 hours at 37°C. BT-474 breast cancer cells were labelled with CellTrace™ Far Red (Thermo Fisher, APC channel) 16 hours prior the experiment. On the day of the experiment, the target cells were pre-incubated with 1 μ g IgE per test for 30 min at 37°C and subsequently co-cultured with RBL SX-38 effector cells (E:T 5:1) for 3.5 hours at 37°C. RBL SX-38 cells were detected using an anti-CD63 antibody and a secondary anti-mouse AF488 antibody. DAPI was added immediately before acquisition on a BD FACSCanto™.

Mast cell degranulation

Rat basophilic leukaemia RBL SX-38 cells were used in the mast cell degranulation assays to measure β -hexosaminidase release, as previously described⁵. Controls were: unstimulated cells; Triton X-100 (100% degranulation); chimeric NIP IgE specific for the hapten 5-iodo-4-hydroxy-3-nitrophenyl (AbD Serotec) in the presence or absence of crosslinking stimulus (polyclonal antigen conjugated to Bovine Serum Albumin (NIP-BSA) or polyclonal rabbit α -human IgE (Dako)). Cells seeded at 1×10^4 cells/well in culture medium overnight were sensitized with IgE (anti-NIP IgE, or anti-HER2 IgE, 200 ng/mL) or left in medium for 1 hour at 37°C, washed three times in HBSS buffer (Hank's Buffered Salt Solution, 1% bovine serum albumin, Invitrogen) and stimulated at 37°C for 30 min with 100 μ L/well antigen (human recombinant HER2 extracellular domain, HER2 ECD), cross-linking agent (polyclonal rabbit α -human IgE, 200 ng/mL), or HER2-expressing SK-BR3 (3×10^4 cells/well) as appropriate. For β -hexosaminidase release detection, 50 μ L culture supernatants diluted 1:1 in HBSS buffer, plus 50 μ L fluorogenic substrate per well (1 mM 4-methylumbelliferyl N-acetyl- β -D-glucosaminide, 0.1% dimethyl sulfoxide

(DMSO), 0.1% Triton X-100, 200 mM Citrate, pH 4.5) were transferred onto black 96-well plate and incubated for 2 hours in the dark. Reactions were quenched with 0.5M Tris (100µl/well) and fluorescence was detected in a Fluostar® Omega microplate reader (350nm excitation, 450nm emission) (BMG Labtech). Degranulation was calculated as % of that measured with addition of Triton X-100 and compared with unstimulated cells (<10%).

Basophil Activation Assay (BAT)

The Basophil activation test (BAT) was carried out using the Flow2 CAST® kit (Bühlmann) according to the manufacturer's instructions. Briefly, unfractionated human blood samples were stimulated, in the presence of stimulation buffer and anti-CCR3/anti-CD63 staining cocktail (Bühlmann), with anti-FcεRI, fMLP (Bühlmann) or anti-human IgE antibody (4.5 µg/ml final concentration; Dako) positive controls, or anti-HER2 or NIP IgE antibodies (3.5 µg/ml final concentration) for 30 minutes at 37°C⁵. Red blood cells were then lysed with 1x lysis solution (Bühlmann) for 10 minutes at room temperature. Samples were centrifuged and cells resuspended in acquisition buffer (Bühlmann). Activation of >500 CCR3^{high}SSC^{low} basophils was assessed as up-regulation of CD63 expression by flow cytometry. Data is expressed as fold change in % CD63 expression upon stimulation, relative to the baseline level when incubated with stimulation buffer alone.

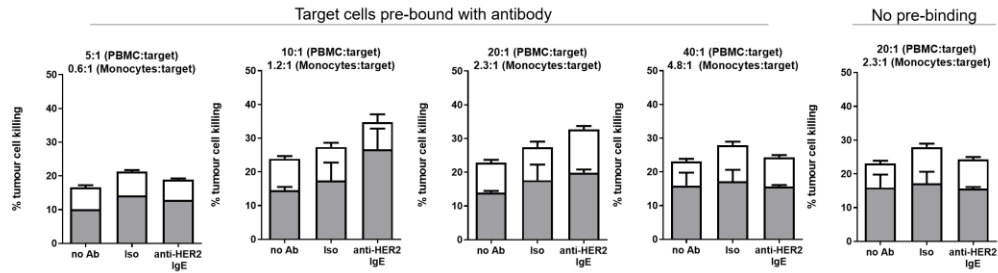
Supplementary References

1. Ilieva KM, Fazekas-Singer J, Achkova DY, et al. Functionally Active Fc Mutant Antibodies Recognizing Cancer Antigens Generated Rapidly at High Yields. *Front Immunol.* 2017;8:1112.
2. Dodev TS, Karagiannis P, Gilbert AE, et al. A tool kit for rapid cloning and expression of recombinant antibodies. *Sci Rep.* 2014;4:5885.
3. Bracher M, Gould HJ, Sutton BJ, Dombrowicz D, Karagiannis SN. Three-colour flow cytometric method to measure antibody-dependent tumour cell killing by cytotoxicity and phagocytosis. *J Immunol Methods.* 2007;323:160-71.
4. Karagiannis P, Singer J, Hunt J, et al. Characterisation of an engineered trastuzumab IgE antibody and effector cell mechanisms targeting HER2/neu-positive tumour cells. *Cancer Immunol Immunother.* 2009;58:915-930.
5. Rudman SM, Josephs DH, Cambrook H, et al. Harnessing engineered antibodies of the IgE class to combat malignancy: initial assessment of FcγεRI-mediated basophil activation by a tumour-specific IgE antibody to evaluate the risk of type I hypersensitivity. *Clin Exp Allergy.* 2011;41:1400-1413.

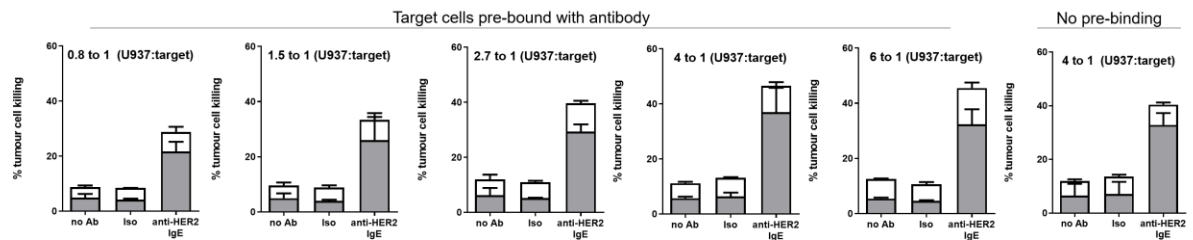
Supplementary Table S1. PIPE PCR primers

PRIMER NAME	SEQUENCE 5'→3'
F-1	CGTACGGTGGCGGCCCATCTGTCTTCATCTTCCCGCCAT
R-1	GGAGTGCGCGCCTGTGGCGGCCGCCACCAAGAAGAGGATC
F-2	GCTAGCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGC
R-2	ACCGCGGCTAGCTGGAACCCAGAGCAGCAGAAACCCAATG
F-HER-VH	GGCCGCCACAGGCGCGCACTCCGAGGTGCAGCTGGTGGAGTCT
R-HER-VH	ACGGATGGGCTCTGTGTGCTAGCTGAGGACACGGTCACCAGAG
F-HER-VL	CTGGGTTCCAGCTAGCCGCGGTGACATCCAGATGACCCAGTCT
R-HER-VL	AGATGGCGCCGCCACCGTACGTTTAATTTCAACTTTGGTACCTTGACC

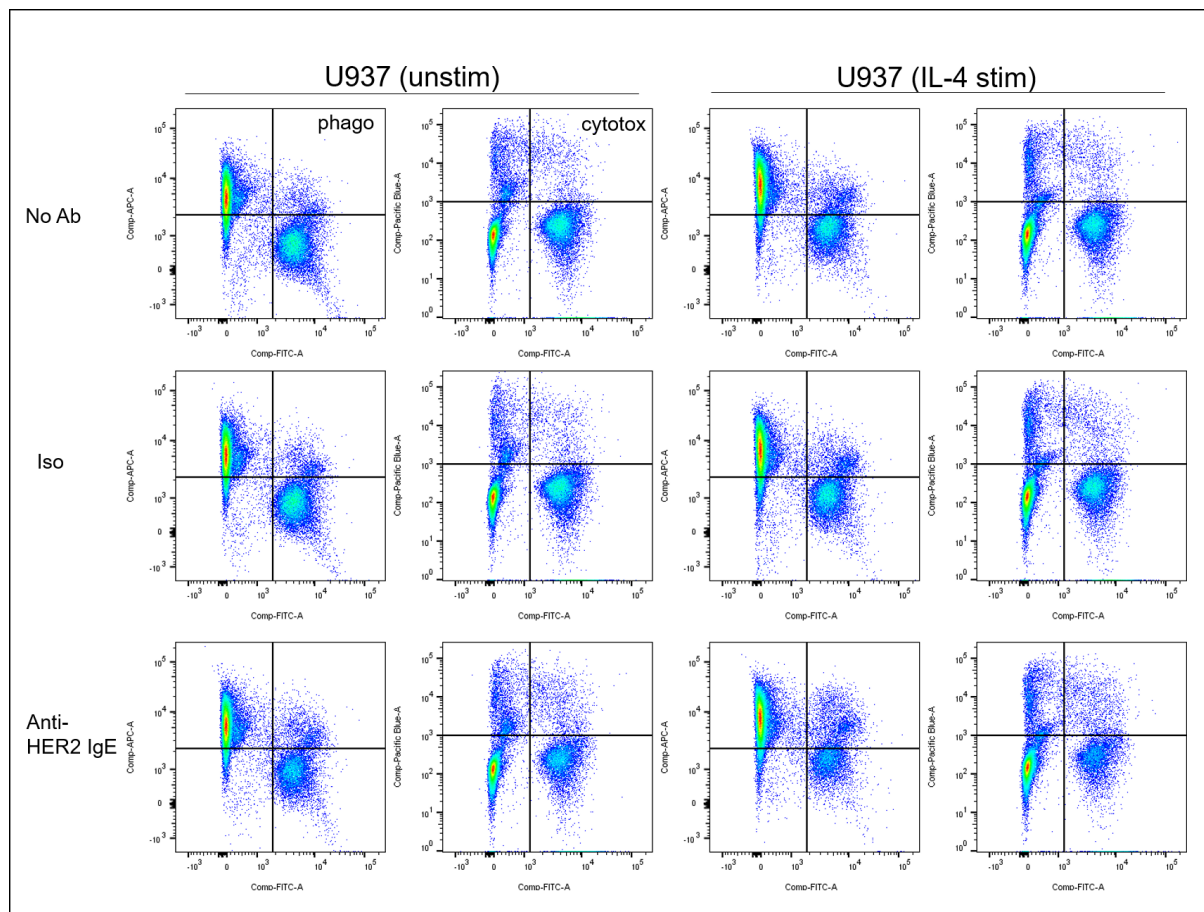
ADCC/ADCP with human healthy volunteer PBMC and BT-474



ADCC/ADCP with U937 and BT-474



Supplementary Figure S1. Optimisation of conditions for the ADCC/ADCP assay. Optimal conditions were established for ADCC/ADCP assays with different effector cell populations: human PBMCs (monocyte effector:target ratios were calculated by flow cytometric analyses) (top panels) and with U937 monocytes (bottom panels). Conditions were established by evaluating different effector:target ratios, and by pre-binding of antibodies to tumour cells, compared to introducing antibody with effector and target cells.



Supplementary Figure S2. ADCC/ADCP assay representative dot plots. Representative dual colour flow cytometric dot plots from which HER2 IgE-mediated anti-tumour phagocytosis (ADCP, left panels) cytotoxicity (ADCC, right panels) and measurements were conducted. In these examples, breast cancer cells were pre-stained with CFSE cell tracking dye (FITC-A), U937 monocytic cells were stained with anti-CD89 APC-conjugated antibody (APC-A), and dead cells were identified using DAPI live/dead dye (Pacific Blue-A). Left panel dot plots depict CFSE+ tumour cells (x-axis) and CD89-PE+ monocytic cells (y-axis) to quantitate total CFSE+ tumour cells and the number of tumour cells present within CD89+ monocytic cells, depicting ADCP by monocytic cells (CFSE+/PE+ cells). Right dot plots depict CFSE+ tumour cells and DAPI+ events (CFSE+/DAPI+ cells), allowing quantitation of tumour targets killed externally (ADCC). Mixed cell populations were incubated with no antibody (No ab), or with non-specific IgE isotype control (iso) or with anti-HER2 IgE. Incubation of HER2/*neu*-expressing breast cancer and U937 cells with anti-HER2 IgE was associated with increased tumour cell death by ADCC.